CHAPTER 12

Corroborative Testing of Viral Isolates

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I. Introduction

Serological testing is an important step in viral identification and confirmation. Several viruses such as IHNV and VHSV can produce similar cytopathic effects on cell cultures making it difficult to determine the identification of the viral agent based on cell culture results alone. Serological tests are highly specific allowing accurate and rapid identification of viruses based on unique antigenic characteristics. The serum neutralization test has long been the standard test for corroboration of viral isolates for these reasons. However, new advances in the past ten years in molecular techniques such as DNA probes and Polymerase Chain Reaction (PCR) allow identification of viral isolates based on their nucleotide sequences. These sequences are used to develop primers specific to the viral strain, or group of viruses. These molecular tools provide an increased sensitivity and unsurpassed specificity that allows rapid and accurate identification of viral isolates.

II. Plaque Reduction Serum Neutralization Assay

Serum neutralization is one method of confirming the serological identity of a virus isolate. When a known concentration of a virus from tissue culture is incubated with a known dilution of specific neutralizing antiserum against that virus, the ability of the virus to then produce CPE when inoculated onto cells is significantly reduced (neutralized). This neutralization is often temporary such that with time the antigen-antibody complex (virus and antibody combined) breaks apart, freeing the virus, allowing it to again infect a cell. This is called "breakthrough" which can confound results if a neutralization test is not read soon enough. Generally, the results of the unknown virus dilutions can be read when the positive control of known virus is significantly neutralized (at least one $\log_{10}$ in titer or 80% plaque reduction).

There are at least two general variations of the virus neutralization test; constant virus Concentration exposed to varying antiserum dilutions or varying virus concentrations exposed to a constant antiserum dilution. The latter type of test requires the least amount of antiserum and has less inherent error in preparation. The following method will apply for those viruses that will produce plaques under a semi-solid overlay (rhabdoviruses, birnaviruses, aquareoviruses, and some herpesviruses).

The plaque reduction serum neutralization assay - can be used to confirm the identity of suspected IHNV isolates from possible viral epizootics or from fish species. Flat bottom 24-well plates are used for this serum neutralization assay.

A. Preparation of plates
    1. Determine number of plates needed for the assay. You will need three 24-well plates to run one unknown virus against one antiserum.
    2. Prepare the plates and allow them to form a monolayer the day before you want to run the assay.

B. Preparing dilutions of known virus, unknown virus, antiserum and normal serum

1. Dilute antiserum to appropriate dilution with MEM. Various dilutions of antiserum will have to be tested against the control virus beforehand to determine the optimum neutralizing dilution. You will need 1.5 ml of diluted antiserum to run one unknown against one antiserum.
2. Dilute normal serum with MEM to the same dilution as the antiserum. You will need 1.5 ml of diluted normal serum to run one unknown against one antiserum.
3. Dilute known virus and unknown virus to approximately 1 x 10^5 pfu/ml with MEM. You will need 1.5 ml of diluted known and unknown virus.

C. Setting up neutralization test and controls
1. Label a sterile unused 24-well plate appropriately, as in the example.
2. Aseptically pipet 200 µl of diluted antiserum into appropriate wells.
3. Aseptically pipet 200 µl of diluted normal serum into appropriate wells.
4. Aseptically pipet 200 µl of MEM into appropriate wells. Add 400 µl of MEM into tissue control well (MEM only).
5. Aseptically pipet 200 µl of each known and unknown virus into appropriate wells.
6. Incubate for one hour at room temperature on a rotary shaker.

EXAMPLE

	A Known Virus	B Known Virus	A Unknown Virus	B Unknown Virus	MEM	
Antiserum	KV + AS	KV + AS	UV + AS	UV + AS	MEM + AS	
Normal Serum	KV + NS	KV + NS	UV + NS	UV + NS	MEM + NS	
MEM	KV + MEM	KV + MEM	UV + MEM	UV + MEM	MEM	

KV Known virus
UV Unknown virus
AS Antiserum
NS Normal serum
MEM Minimum essential media

D. Performing the assay
1. Label the three 24-well plates to be used in the plaque assay. Three duplicate tests are run on one plate, i.e., plate 1 may contain:

e.g., KV + AS; UV + AS; KV + NS

KV + AS	A	10^{-0}	10^{-1}	10^{-2}	10^{-3}
	B	10^{-0}	10^{-1}	10^{-2}	10^{-3}
UV + AS	A	10^{-0}	10^{-1}	10^{-2}	10^{-3}
	B	10^{-0}	10^{-1}	10^{-2}	10^{-3}
KV + NS	A	10^{-0}	10^{-1}	10^{-2}	10^{-3}
	B	10^{-0}	10^{-1}	10^{-2}	10^{-3}

The antiserum, normal serum and MEM controls can be run on the third plate using only 10^{0} and 10^{-1} dilutions.

2. Dilute 0.1 ml of the solution from each test well in the incubated 24-well plate 10^{-0} to 10^{-3} in 0.9 ml MEM dilution blanks. Dilute 0.1 ml of the mixture from each control well 10^{-0} to 10^{-1} in 0.9 ml MEM dilution blanks.
3. Overlay EPC cells with 100 µl of 7% PEG for a few minutes prior to inoculations. PEG solution should be made up in MEM-10.
4. Pipet 100 µl of each dilution into appropriate well of PEG-treated EPC cells.
5. Incubate for 30 minutes at room temperature to allow virus adsorption.
6. Overlay wells with 1 ml of methylcellulose overlay medium.
7. Incubate at 15°C for 7 days in a sealed plastic bag or plastic container.
8. Fix and stain plates by pipetting approximately 1 ml of 0.5% crystal violet in 40% formalin into each well and let stand for 1 hour.
9. Pour off stain, rinse monolayers with water and allow plates to air dry.
10. Count and record numbers of plaques.

E. Interpretation

1. The tissue control wells (MEM only), the AS + MEM wells and the NS + MEM wells should not have any plaques present. Plaques would indicate that the medium, antiserum or normal serum were contaminated with virus and the test must be repeated.
2. An 80% or greater reduction of plaques is considered a positive serum neutralization test and confirms the identity of the virus. The known virus control should always show an 80% or greater reduction for the test to be valid.
3. When determining if there is an 80% reduction of plaques, first look at the virus control wells. Determine the dilution where countable numbers of plaques are present. Calculate the mean pfu of the duplicate wells and compare this value to that of the virus + AS wells at that same dilution. Subtraction of the latter value from the control value will provide the pfu/ml of virus remaining after neutralization.
    a. Calculating pfu/ml: Viral titer for each sample is expressed as mean plaque-forming units (pfu) per ml of test tissue or per gram of tissue. The best wells to use for determining titer are those of the highest dilutions with between 20 and 200 plaques.
    b. The following equation is used to express pfu/ml (or gram of tissue) in one well:

plaques x 1/tube dilution x 1/# ml added to well.

e.g. The 10^{-4} well of ovarian fluid sample A has 20 plaques in the cell monolayer.
1/tube dilution = $1/10^{-4} = 10^4$
1/# ml = 1/0.1 ml = $1/10^{-1} = 10^1$
20 plaques x 10^4 x 10^1 = 2.0 x 10^6 pfu/ml ovarian fluid

4. The normal serum + virus wells should not show any plaque reduction as compared to the virus positive MEM wells. If there is significant plaque reduction in the normal serum wells, this indicates that there is some nonspecific neutralization occurring with the virus and the serum.

   Another method of expressing neutralization is the neutralization index (NI). This value is calculated by subtracting the $\log_{10}$ pfu/ml value of the neutralized virus remaining from the value of the same un-neutralized virus in MEM. Example:

Antisera	$\log_{10}$ pfu/ml Remaining Virus			
	IHNV	**NI**	VHSV	**NI**
IHNV	2.0	**2.8**	4.4	**0.4**
VHSV	4.8	**0**	<1.0	**>3.8**
MEM	4.8	**0**	4.8	**0**

   Using Log_{10} pfu, a smaller value denotes greater neutralization.

   After conversion to NI the opposite is true, i.e., the larger the NI value the greater the neutralization.

III. Immunoblot

Dot blot is a relatively simple and quick assay to differentiate virus detected in cell culture; however, it should be employed as an additional test for viral identification. Serum neutralization or PCR is still suggested for confirmation.

Run controls for each assay using known virus as positive controls; cell cultures and PBS as negative controls. Follow good laboratory techniques by handling all suspect samples as positive for virus. Discard capillary tubes, wash solutions, and all supplies coming in contact with samples into an appropriate biohazard container or a chlorine solution.

NOTE: Heat-inactivated IHN and VHS viruses (60°C for 2 hours) work well in dot blot and eliminate the need for biohazard precautions.

A. BLOTTING PROCEDURES

1. Don't touch the nitrocellulose membrane (paper) with hands. Use forceps and handle the paper by the edges.

2. Draw a grid of 1 cm squares on the paper using a permanent alcohol/waterproof marker.

Cut into strips; one strip will be used for each antisera.

3. Pour PBS 1X into a glass staining dish. Slide one edge of strip just under the surface of the PBS and slowly submerge paper until uniformly wet. After soaking for a few minutes, remove strips and air dry on bibulous paper for 5 minutes.

4. Make a map of your sample placement. With microcapillary tubes, slowly spot 10µl of each sample on a square of the paper. Air dry until paper appears completely dry (approximately 5-10 minutes). If using two antisera, repeat this process, in the same order, on the second antisera strip.

5. In a glass staining dish, immerse the strips for 20 minutes in the 3% gelatin solution. Keep at 37°C to prevent gelatin from solidifying.

6. Remove each strip and quickly rinse in PBS 1X to remove excess gelatin. Then place each strip in a plastic Ziploc bag or "seal-a-meal" pouch and add 3-5 ml of the antisera of choice (IHN, IPN, or VHS). Seal and incubate at 37°C for 60 minutes. Agitate (by palpating pouch) every 15 minutes.

6. The primary antibody is saved for reuse. Open sack and carefully remove all antisera, making sure antisera is returned to its appropriate container.

7. Place the paper in a glass dish with Tween 20 solution (TPBS) and wash for 30 minutes, changing the TPBS every 10 minutes. The TPBS should be approximately 3-4 cm deep for each wash. Use a separate dish for each antisera used.

8. Put paper strips in a plastic Ziploc bag or "seal-a-meal" pouch and add 5-10 ml of the secondary antibody [goat anti-rabbit IgG conjugated with horseradish peroxidase (GAR-HRP)]. Incubate for 60 minutes at 37° C. Agitate every 15 minutes.

10. Open sack and discard the second antibody solution. Wash strips as in step 8 above.

B. COLOR DEVELOPMENT

1. Prepare solutions A and B at room temperature during the final wash. Mix the solutions immediately prior to use.

2. Put paper in a glass dish, add the color developer solution, and place in the dark. Allow to develop 7-10 minutes.

3. Remove strips from the developer and wash in running water for 15 minutes. Positive reactions will appear as dark blue spots where samples were blotted. Place strips between two 96-well plate sealers if you wish to keep results as reference or records.

C. FORMULAS FOR DOT BLOT REAGENTS

PBS 10X (Calcium & Magnesium Free) - Indefinite shelf life @ room temperature

NaCl	80.0 gm
KCl	2.0 gm
Na_2HPO_4	11.5 gm
KH_2PO_4	1.0 gm
Thimerosal (0.01%)	0.1 gm

qs to 1000 ml with cell culture water

PBS 1X - Shelf life at least 1 month @ room temperature

Cell culture water	900.0 ml
PBS 10X (Ca^{++}, Mg^{++} free)	100.0 ml

3% Gelatin - Shelf life stable for at least 1 month @ 4°C

Gelatin [Enzyme Immunoassay (EIA) Grade]	3.0 gm
PBS 1X	100.0 ml

Heat or microwave to dissolve gelatin.

Tween 20 Solution (TPBS) - Shelf life at least 1 month @ room temperature

PBS 1X	500.0 ml
Tween 20	0.1 ml (5 drops)

Primary Antibody – Recommended dilution for the primary antibody (i.e., rabbit antisera against IHN, IPN, or VHS) is 1:1000.

Secondary Antibody - Goat anti-Rabbit Horseradish Peroxidase Conjugate (GAR-HRP)

	1:2000	1:3000
GAR-HRP	10.0 µl	10.0 µl
PBS 1X	20.0 ml	30.0 ml

Color Development Solution (CDS)

Solution A - HRP Color Development Reagent	30.0 mg (0.03 gm)
Cold methanol (reagent grade)	10.0 ml
Solution B - Cold hydrogen peroxide (30% stabilized reagent grade) (Recommend J. T. Baker, cat. #JT-2186-1, 500 ml.)	30.0 µl
PBS 1X	50.0 ml

Prepare A and B solutions during final wash period, prior to color development. Mix A and B solutions together immediately prior to use to make color development solution.

IV. Indirect Fluorescent Antibody Staining (IFAT) for Viral Identification

Immunofluorescence assay for IHNV - is used as another rapid serological test for confirming the identity of a viral isolate as IHNV. The assay uses a primary mouse anti-IHNV monoclonal

antibody and a goat anti-mouse IgG FITC conjugate. The assay is performed in microwells of standard FAT slides

A. MATERIALS

cleaned FAT slides and coverslips (Freed, Inc.)
disposable petri dishes
gauze swabs
airtight plastic container
filtered (0.45 μm) distilled water
MEM-10-3X (3x antibiotic)
EPC cells
MAb mouse monoclonal anti-IHNV antiserum (or other suitable antiserum)
Goat anti-mouse IgG FITC conjugate (Cappel)
known and unknown virus isolates

B. SETUP

1. Work is done in a tissue-culture hood.
2. Clean FAT slides in 70% alcohol and wipe with gauze swabs.
3. Place the clean slides in the sterile petri dishes (chambers), 1 slide per dish. Cut 2 strips from a gauze swab and lay in the dish alongside the slide. (Soak the gauze with distilled water for humidity.)
4. Seed each of the necessary wells with EPC cells in MEM-10-3X of sufficient density to monolayer overnight (1 drop per well from 5-ml pipet). Put all chambers into an airtight container for incubation @ 21^{o}C overnight.

C. SAMPLE INOCULATIOIN AND INCUBATION

1. When cells are ready, the medium is dumped from each slide into a waste beaker containing bleach.
2. Add a tissue-culture isolate of suspected IHNV to cells, 1 drop per well. Replicate slides are prepared, one for each incubation period; - 8, 12, 24 and 48 hours. The suspect IHNV isolate should be taken from a culture having 3-4+ CPE.
3. Replicate controls are prepared at the same time, on different slides, for identical treatment and incubation periods. Place experimental and control slides each in separate chambers @ 15^{o}C for the prescribed times. Controls should include: a known IHNV isolate stained with and without the primary antiserum (these could be on the same slide) followed by the conjugate; uninfected cells stained with all reagents.

For a single tested isolate there would be: 4 slides of cells infected with unknown virus, each in a single chamber for the 4 incubation periods; 4 slides of cells infected with known IHNV in another chamber; and 4 slides of uninfected cells in a third chamber. Hence, there would be 3 slides (unknown, known, uninfected) removed for staining at each incubation interval. The optimum sample will be the incubation interval just prior to early CPE.

D. FIXATION

1. At scheduled incubation periods, an experimental and the 2 control slides are washed for 5 minutes in a Coplin jar containing cold PBS.
2. Slides are fixed in methanol for 10 minutes.
3. Slides can be stored @ 4°C until ready to stain.

E. STAINING

1. Undiluted MAb is added to appropriate wells (except negative IHNV control) and allowed to incubate for 5 minutes.
2. Rinse slides in 0.45 µm filtered distilled water for 5 minutes and gently shake free of water.
3. Goat anti-mouse conjugate diluted 1:160 (or as determined) is added to each well for 5 minutes.
4. Rinse in filtered distilled water for 5 minutes as above and shake off excess water.
5. Remove and coverslip with a minimum amount of FA mounting fluid and observe at 1000X for cytoplasmic fluorescence in the known positive control. No fluorescence should be observed in the negative controls. Read results for the unknown samples.

V. Biotinylated DNA Probes for Detection of IHNV and Distinction Between the European and North American Strains of VHSV

Introduction: The DNA Probe detects and identifies isolates of IHNV, North American VHSV, and European VHSV, using the one-day dot blot procedure from infected fish cell cultures

Biotinylated DNA probes for IHNV and both European and North American strains of VHSV were developed by Deering et al.(1991) and by Batts et al. (1993). The probes hybridize with different sequences within the messenger RNAs of the nucleoprotein (N) gene elicited by each of the viruses that is extracted from tissue culture cells that have been infected for 24-48 hours. The probe for North American VHSV hybridizes specifically with a nearly unique 28-nucleotide sequence following the open reading frame of the N gene mRNA. The probe recognizing all strains of VHSV binds to a 29-nucleotide sequence near the center of the N gene common to both American and European strains. The IHNV-specific probe (Deering et al. 1991) recognizes a 30 base sequence unique only to IHNV. The following procedure was adapted from Batts et al. (1993) who have graciously supplied their protocol.

A. DNA PROBE SET UP

Two days before running DNA Probe Test:

1. Prepare a 24-well microtiter plate with EPC or CHSE-214 cells so that it will be confluent by the next day.

2. If necessary, make up DEPC-treated water at a concentration of 1 ml DEPC to 1 L of distilled water. Mix on stir plate until thoroughly mixed. Make at least 5-6 L for treating glassware.

3. Rinse needed glassware with DEPC treated water and let dry. Store on shelf in an area dedicated for this use.

4. Sterilize distilled water and make up solutions that need to be autoclaved (solutions f, g, l, m, n).

One day before running DNA probe test:

1. Inoculate viral isolates onto cell monolayers in 24-well plate. Use several wells per isolate. Inoculate 2 wells with MEM-10-TRIS to use as a negative control. There should be no CPE when mRNA is extracted. Use dilutions if CPE occurs in 24-48 hrs. Incubation of virus on cells may require up to 48 hrs for adequate mRNA from certain isolates.
2. Prepare all other solutions needed to run the test. Adjust pH of final products carefully.
3. Fill both water baths and turn on. Adjust to 55°C and 65°C.

4. Get out rotator, Hybridot, and Seal-a-Meal and make sure they are operational.

B. DNA PROBE TEST PROCEDURE

1. Extraction of mRNA from infected cells:
    a. Preparation
        (1) Place crushed ice in a tray with microcentrifuge racks.
        (2) Make sure water baths are at 55°C and 65°C.
        (3) Always wear latex gloves.
        (4) Label tubes to be used.
    b. Pipet off the infectious medium above cells and add 0.5 ml RNAzol B to each well. Replace lid and put on rocker for 5-10 minutes at room temperature to digest cells.
    c. During step b put 50 µl cold chloroform/iso-amyl alcohol into labeled siliconized 1.7-ml tubes and keep on ice.
    d. Triturate the cell debris in each well with a 1-ml pipet five times and transfer solution into the labeled chloroform/iso-amyl tubes. Vortex the tubes 3 seconds each and store on crushed ice for 5 minutes to allow phase separation.
    e. Centrifuge the suspension at 10,000 rpm for 15 minutes. The RNA will remain in the clear aqueous phase and the DNA and protein will be left in the lower blue phenol phase.
    f. During step e, put 0.25 ml of cold absolute isopropyl alcohol into new labeled tubes and store on ice. Keep the alcohol at -20°C until ready for use.
    g. Transfer the aqueous phase containing the RNA (0.25 ml, no blue fluid) into the tube with 0.25 ml absolute isopropyl alcohol. Vortex for 1 second and chill tubes on ice for 15 minutes to precipitate RNA.
    h. Centrifuge for 15 minutes at 10,000 rpm and remove as much fluid as possible from pellet. When you centrifuge, put the hinge of the microtube on the top. The pellet will be on that side and may be very difficult to see.
    i. During step h, prepare nitrocellulose membrane:

(1) Wet membrane in distilled water for 1 minute. Wet by capillary action at an angle.
(2) Pour water off.
(3) Soak for at least 5 minutes in 10X standard saline citrate (SSC).

i. For each probe used, heat approximately 140 µl of North American VHSV, Common VHSV and IHNV PCR products for 1 minute in boiling water to denature the DNA. Transfer to ice. If only two probes are used, heat about 250 µl of product.
k. Warm prehybridization buffer to 55°C in water bath.
l. Add 170 µl autoclaved distilled water to RNA pellets. Mix by flicking bottom of tube and warm tubes in 65°C water bath for 15-20 minutes. RNA pellets should dissolve. Mix again. Pellets appear as small white or brown flakes.
m. Add 170 µl of 20X SSC into tubes containing dissolved RNA pellets and store on ice.
n. During step k put wetted membrane in Hybridot. Attach vacuum pump hoses to blotting device.
o. Add 200 µl of 10X SSC to each well of blotting device. Membrane should not be dry when RNA is added. Try to avoid trapping air in the wells of the Hybridot.
p. Mix gently and add 100 µl of each RNA solution to wells of Hybridot which contain 200 µl of 10X SSC. Blot PCR products last.
q. Apply vacuum at 5 psi. After all solutions are added leave vacuum on 10-15 psi for about 1 minute. Turn off vacuum. Poke holes with pipet tip into empty wells for easy cutting of membrane.
r. Dismantle apparatus and remove membrane with forceps. Transfer membrane to thick filter paper wetted with 10X SSC.
s. Cut membrane into sections and label.
t. Transfer membranes to dry sheet of blotting paper and cover with a second sheet. Microwave for 60 seconds on high to attach nucleic acids to membrane. Weights can be placed on sides of the blotting paper to keep it from curling up.

2. Hybridization of probes with RNA on nitrocellulose membrane:
   a. For prehybridization, place membranes spot-side-up into separate Seal-A-Meal® pouches. Add 10 ml prehybridization buffer to each pouch, remove air bubbles, and seal. Prehybridize for 30 minutes to 24 hours at 55°C in water bath.
   b. Thaw the probe solutions and heat to 50-55°C. Cut off edge of pouches and pour off the prehybridization buffer. Add 10 ml of each probe solution (prediluted in buffer) to the respective pouch and re-seal. React membranes in probe solutions for 1 hour to 24 hours at 55°C in water bath. If you are using probes that are not prediluted, do not pour off the prehybridization buffer and add 100 µl of probe.
   c. Remove probe solutions from pouches and store in tubes at -20°C for reuse up to 5 times.
   d. Transfer membranes into 40 ml post-hybridization solution in a buffer dish. Wipe forceps between each membrane. Discard solution and add 40 ml fresh post-hybridization solution and wash for 15 minutes on rocker at RT. Wash two more times with 40 ml buffer for 15 minutes each on the rocker at RT.
   e. Put dish with membranes and pre-warmed post-hybridization buffer into 55°C

waterbath for 15 minutes. Cover dish with parafilm.

f. Warm color development buffer to RT.
g. Rinse membranes briefly with 40 ml of Buffer A.

3. Color development of biotinylated probe:
   a. Incubate membranes in a solution containing 40 µl streptavidin/alkaline phosphatase conjugate in 40 ml Buffer A for 30 minutes on rocker at RT. The conjugate can be used up to five times.
   b. Rinse membranes briefly in 40 ml Buffer A and then wash twice in 40 ml Buffer A on the rocker for 7 minutes at RT.
   c. Wash twice in 40 ml Buffer B on the rocker for 7 minutes at RT.
   d. Immediately before use, add 0.4 ml alkaline phosphatase (AP) color reagent A and 0.4 ml AP color reagent B to 39.2 ml color development buffer warmed to RT.
   e. Add 40 ml color development solution to the dish containing the membranes. Store in the dark for 15 minutes on the rocker at RT. The rocker can be placed under a box for this step.
   f. Wash membranes in distilled water for 10 minutes with at least one change of water. Store membranes in distilled water until ready to photograph.

4. Probes for IHNV and VHSV are synthesized from the sequences given in Derring et al. (1991) or Batts et al. (1993). They can be obtained from the Western Fisheries Research Center in Seattle.

5. Solutions needed for DNA probe dot blot procedures:

   All glassware should be Cleaned with DEPC-treated water and autoclaved before use. This is to prevent RNA-ase contamination. This water is available from Five Prime→Three Prime, Inc. (catalog #5302-336550)

(a) **PREHYBRIDIZATION BUFFER**

Distilled-deionized water	69.5 ml
10x Denhardt's solution	10 ml of 100x stock
2x SSC	10 ml of 20x stock
1% SDS	10 ml of 10% stock
0.1 mg/ml SSS DNA (Five Prime→Three Prime)	0.5 ml of 20 mg/ml stock

(b) **HYBRIDIZATION SOLUTION**

Prehybridization buffer	10 ml
Biotinylated DNA probe	100 ng/ml

(Store at -20°C; may reuse up to 5 times)

(c) **POST-HYBRIDIZATION SOLUTION**

2x SSC	50 ml of 20x stock
0.1% SDS	5 ml of 10% stock
distilled-deionized water	up to 500 ml

(d) **DENHARDT'S SOLUTION**

(Commercial product (Five Prime→Three Prime, Inc.) purchased at 100x stock concentration)
(Catalog #5302-213502 for 100-ml size)

A 10x solution contains:
1% bovine serum albumin
1% polyvinylpyrrolidone 360
1% ficoll 400

(e) **SONICATED SALMON SPERM DNA (SSS DNA)**

(Commercial product (Five Prime→Three Prime, Inc.) purchased at 20 mg/ml)
(Catalog #5302-754688 for 5-ml size)

Procedure
Transfer 0.5 ml of SSS DNA into 10 vials (with gaskets). Place vials into boiling water for 10 minutes. Cool vials in crushed ice, then transfer to -20°C freezer until needed. When needed, add 0.5 ml to prehybridization buffer (see #1)(final concentration of 0.1 mg/ml).

(f) **20x STANDARD SALINE CITRATE (20X SSC)**

NaCl (Sigma #S-3014, 3 M final concentration)	87.65 g
citric acid (Sigma #C-8532, 0.3 M final concentration)	44.11 g
distilled-deionized water	up to 500 ml

(Adjust to pH 7.0 with HCl, AUTOCLAVE)
(Option: may purchase product already prepared from Five Prime→Three Prime, Inc.)
(Catalog #5302-227160)

(g) **10x STANDARD SALINE CITRATE (10X SSC)**

NaCl (Sigma #S-3014, 3 M final concentration)	43.82 g
citric acid (Sigma #C-8532, 0.3 M final concentration)	22.05 g
distilled-deionized water	up to 500 ml

(Adjust to pH 7.0 with HCl, AUTOCLAVE)
OR
Dilute 1:2 from 20x SSC. Combine equal volumes of 20x SSC with distilled-deionized water, AUTOCLAVE.

(h) **10% SODIUM DODECYL SULFATE (10% SDS)**

Lauryl sulfate sodium salt (Sigma #4390)	10.0 g
sterile distilled-deionized water	up to 100 ml

(Adjust to pH 7.2. **Do not autoclave this solution!**)

(i) **STREPTAVIDIN/ALKALINE PHOSPHATASE CONJUGATE (SA/AP)**

0.1 µg/ml streptavidin/alkaline phosphatase conjugate (BRL #9543SA), store vial at 4°C.

Prepare by diluting SA/AP 1:1000 in Buffer A:
(Example: 30µL SA/AP stock added to 30 ml of Buffer A)

(May reuse this solution up to 5 times, store at 4°C)

(j) **BUFFER A**

0.1 M Tris (pH 7.5)	50 ml of 1 M stock (#14)
0.1 M NaCl	10 ml of 5 M stock (#12)
2 mM $MgCl_2$ (Sigma #M-1028, 100 ml size)	1 ml of 1 M stock
0.05% Triton X-100 (BIORAD, Catalog #161-0407)	0.25 ml
distilled-deionized water	up to 500 ml

(k) **BUFFER B**

0.1 M Tris (pH 9.5)	50 ml of 1 M stock (#13)
0.1 M NaCl	10 ml of 5 M stock (#12)
50 mM $MgCl_2$ (Sigma #M-1028, 100 ml size)	25 ml of 1 M stock
distilled-deionized water	up to 500 ml

(l) **5 M NaCl**

NaCl (Sigma #S-3014)	146.1 g
distilled-deionized water	up to 500 ml

(AUTOCLAVE THIS SOLUTION)

(m) **1 M TRIS BUFFER (pH 9.5)**

Tris base (Sigma #T-8524)	54.7 g
Tris HCl (Sigma #T-7149)	7.6 g
distilled-deionized water	up to 500 ml

(Adjust to pH 9.5, then AUTOCLAVE!)

(n) **1 M TRIS BUFFER (pH 7.5)**

Tris base (Sigma #T-8524)	11.8 g
Tris HCl (Sigma #T-7149)	63.5 g
distilled-deionized water	up to 500 ml

(Adjust to pH 7.5, then AUTOCLAVE!)

(o) **CHLOROFORM/ISOAMYL ALCOHOL MIXTURE**

chloroform (J.T. Baker #9180-03)	24 ml
iso-amyl alcohol (J.T. Baker #9038-1)	1 ml

(Mix together and store at -20°C until needed)

(p) **ISOPROPYL ALCOHOL**

2-Propanol (isopropyl alcohol), (J.T. Baker #9084-03),

(Use undiluted for precipitation of RNA)

(q) **RNAzol B**

RNA isolation solvent, store at 2-8°C in dark. Cinna Biotecx Laboratories, Inc. 6023 South Loop East, Houston, Texas 77033. 1-800-535-6286. Catalog #104B is 100-ml size. Contains guanidine thiocyanate, 2-mercaptoethanol, and phenol.

(r) **ALKALINE PHOSPHATASE CONJUGATE SUBSTRATE KIT**

(**NOTE:** This product contains dimethylformamide. Use in area with good ventilation.)
(BIORAD Catalog #170-6432)

1. Dissolve AP color development buffer in 1 L volume of distilled-deionized water.
2. Filter-sterilize then store at 4°C until needed.
3. Immediately before use, add 0.3 ml of AP color reagent A and 0.3 ml AP color reagent B to 29.4 ml color development buffer at RT.

RAPID DOT BLOT (7 h)

CELLS INFECTED WITH VHSV OR IHNV

8:30

9

EXTRACT RNA (2 h)

10:30

9

BLOT RNA, GET READY TO PREHYBRIDIZE (0.5 h)

11:00

9

PREHYBRIDIZE MEMBRANE (1 h)

12:00

9

HYBRIDIZE TARGET RNA ON MEMBRANE IN PROBE SOLUTION (1 h)

1:00

9

COLOR DEVELOPMENT OF SPOTS (2.5 h)

3:30

VI. PCR for Detection of Fish Viruses

A. PCR FOR INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

1. Viral RNA Preparation

Release RNA from virus solution or tissue homogenate by diluting sample 1:20 in sterile deionized water, heating tube at 95˚C for 2 min, then storing on ice.

2. Reverse Transcription and 1st Round PCR Protocol

a. Prepare a master mix for the number of samples to be analyzed. Work under a hood and wear gloves.

b. The master mix for one 50 µL reverse transcription PCR is prepared as follows:
   1) 23.75 µL DEPC water
   2) 5 µL 10 X Buffer
   3) 5 µL 25 mM $MgCl_2$
   4) 5 µL 2 mM dNTP
   5) 2.5 µL (20 pmoles/µL) Upstream Primer
      5'-TCA AGG GGG GAG TCC TCG A-3', Tm 62˚C
   6) 2.5 µL (20 pmoles/µL) Downstream Primer
      5'-CAC CGT ACT TTG CTG CTA C-3', Tm 58˚C
   7) 0.5 µL Taq Polymerase (5 U/µL)
   8) 0.5 µL AMV reverse transcriptase (9 U/µL)
   9) 0.25 µL RNasin (39 U/µL)

c. Dispense 45 µL of the master mix into each tube and add 5 µL of template RNA. Be sure to include the following:
   i) Positive control, IHNV or purified IHNV RNA
   ii) Negative control, no RNA

d. Centrifuge the tubes briefly (10 sec) to make sure the contents are at the bottom.

e. Place the tubes in the thermal cycler and start the following cycles:
   i) 1 cycle - 50˚C for 15 min
   ii) 1 cycle - 95˚C for 2 min
   iii) 25 cycles - 95˚C for 30 sec, 50˚C for 30 sec, 72˚C for 60 sec
   iv) 1 cycle - 72˚C for 7 min and soak at 4˚C

f. Visualize the 786 bp PCR amplicon by electrophoresis of the product in 1.5% agarose gel with ethidium bromide and observe using UV transillumination.

3. Second Round PCR Protocol

a. If the first round PCR provides insufficient amplified product a nested set of primers is used for additional DNA amplification. Prepare a master mix for the number of samples to be analyzed. Work under a hood and wear gloves.

b. The master mix for one 50 µL 2nd round PCR is prepared as follows:
   1) 27.5 µL DEPC water
   2) 5 µL 10 X Buffer
   3) 5 µL 25 mM $MgCl_2$
   4) 5 µL 2 mM dNTP
   5 2.5 µL (20 pmoles/µL) Upstream Primer
      5'-TTC GCA GAT CCC AAC AAC AA-3', Tm 58˚C
   6) 2.5 µL (20 pmoles/µL) Downstream Primer
      5'-GCG CAC AGT GCC TTG GCT-3', Tm 60˚C
   7) 0.5 µL Taq Polymerase (5 U/µL)

c. Dispense 48 µL of the master mix into each tube and add 2 µL of 1st round PCR template.

d. Centrifuge the tubes briefly (10 sec) to make sure the contents are at the bottom.

e. Place the tubes in the thermal cycler and start the following cycles:
   i) 1 cycle - 95˚C for 2 min
   ii) 25 cycles - 95˚C for 30 sec, 50˚C for 30 sec, 72˚C for 60 sec
   iii) 1 cycle - 72˚C for 7 min and soak at 4˚C

f. Visualize the 323 bp PCR amplicon by electrophoresis of the product in 1.5% agarose gel with ethidium bromide and observe using UV transillumination.

There is a DNA probe procedure that can be used to further confirm that the amplicon is from IHNV.

Reference

Deering RE, CK Arakawa, KH Oshima, PJ O'Hara, ML Landolt, and JR Winton. 1991. Development of a biotinylated DNA probe for detection and identification of infectious hematopoietic necrosis virus. Dis. Aquat. Org. 11: 57-65.

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NOTE: Other primers have also been shown to be successful; however, these were selected since they are conserved at the nucleotide level for all known isolates of IHNV and are not conserved for the closely related fish rhabdovirus, VHSV.

B. PCR FOR VIRAL HEMORRHAGIC SEPTICEMIA VIRUS

1. Viral RNA Preparation

Release RNA from virus solution or tissue homogenate by diluting sample 1:20 in sterile deionized water, heating tube at 95˚C for 2 min, then storing on ice.

2. Reverse Transcription and 1st Round PCR Protocol

a. Prepare a master mix for the number of samples to be analyzed. Work under a hood and wear gloves.

b. The master mix for one 50 µL reverse transcription PCR is prepared as follows:
   1) 23.75 µL DEPC water
   2) 5 µL 10 X Buffer
   3) 5 µL 25 mM $MgCl_2$
   4) 5 µL 2 mM dNTP
   5) 2.5 µL (20 pmoles/µL) Upstream Primer
      5'-TCT CTC CTA TGT ACT CCA AG-3', Tm 58˚C
   6) 2.5 µL (20 pmoles/µL) Downstream Primer
      5'-TTC CGG TGG AGC TCC TGA AG-3', Tm 64˚C
   7) 0.5 µL Taq Polymerase (5 U/µL)
   8) 0.5 µL AMV reverse transcriptase (9 U/µL)
   9) 0.25 µL RNasin (39 U/µL)

c. Dispense 45 µL of the master mix into each tube and add 5 µL of template RNA. Be sure to include the following:
   i) Positive control, VHSV or purified VHSV RNA
   ii) Negative control, no RNA

d. Centrifuge the tubes briefly (10 sec) to make sure the contents are at the bottom.

e. Place the tubes in the thermal cycler and start the following cycles:
   i) 1 cycle - 50˚C for 15 min
   ii) 1 cycle - 95˚C for 2 min
   iii) 25 cycles - 95˚C for 30 sec, 50˚C for 30 sec, 72˚C for 60 sec
   iv) 1 cycle - 72˚C for 7 min and soak at 4˚C

f. Visualize the 950 bp PCR amplicon by electrophoresis of the product in 1.5% agarose gel with ethidium bromide and observe using UV transillumination.

3. Second Round PCR Protocol:

a. If the first round PCR provides insufficient amplified product a nested set of primers is used for additional DNA amplification. Prepare a master mix for the number of samples to be analyzed. Work under a hood and wear gloves.

b. The master mix for one 50 µL 2nd round PCR is prepared as follows:

1) 27.5 µL DEPC water
2) 5 µL 10 X Buffer
3) 5 µL 25 mM $MgCl_2$
4) 5 µL 2 mM dNTP
5) 2.5 µL (20 pmoles/µL) Upstream Primer
   5'-ATG GGC TTC AAG GTG ACA C-3', Tm 58˚C
6) 2.5 µL (20 pmoles/µL) Downstream Primer
   5'-GTA TCG CTC TTG GAT GGA C-3', Tm 58˚C
7) 0.5 µL Taq Polymerase (5 U/µL)

c. Dispense 48 µL of the master mix into each tube and add 2 µL of 1st round PCR template.

d. Centrifuge the tubes briefly (10 sec) to make sure the contents are at the bottom.

e. Place the tubes in the thermal cycler and start the following cycles:
   a) 1 cycle - 95˚C for 2 min
   b) 25 cycles - 95˚C for 30 sec, 50˚C for 30 sec, 72˚C for 60 sec
   c) 1 cycle - 72˚C for 7 min and soak at 4˚C

f. Visualize the 558 bp PCR amplicon by electrophoresis of the product in 1.5% agarose gel with ethidium bromide and observe using UV transillumination.

There is a DNA probe procedure that can be used to further confirm that the amplicon is from VHSV.

References

Batts WN, CK Arakawa, J Bernard, and JR Winton. 1993. Isolates of viral hemorrhagic septicemia virus from North America and Europe can be detected and distinguished by DNA probes. Dis. Aquat. Org. 17: 67-71.

Einer-Jensen K, NJ Olesen, N Lorenzen, PEV Jorgensen. 1995. Use of the polymerase chain reaction (PCR) to differentiate serologically similar viral hemorrhagic septicemia (VHS) virus isolates from Europe and America. Vet Res 26: 464-469.

NOTE: Other primers have also been shown to be successful; however, these were selected since they are conserved at the nucleotide level for all known isolates of VHSV and are not conserved for the closely related fish rhabdovirus, IHNV.

C. PCR FOR LARGEMOUTH BASS VIRUS

Protocols for confirmation of Largemouth Bass Virus developed by John A. Plumb et al. 1999 (Journal of Aquatic Animal Health 11:391-399). This protocol was adapted by Becky Lasee and Brian Trewyn of the Lacrosse Fish Health Center, Lacrosse, Wisconsin.

1. Materials and Reagent

Qiagen DNeasy Tissue kit (Qiagen #69506)
Ethanol (Absolute 97-100%) Proteinase K (found in Qiagen kit)
Lysozyme Lysis Buffer
Pipetter (0.5-10 µl, 10-100 µl, 100-1000 µl)
Aerosol Barrier Tips
1.5 ml centrifuge tubes (autoclaved)
Microcentrifuge
Incubator (37°C)
Dry heating bath (55°C & 70°C)
Latex or nitrile gloves

2. Sample Collection and Extraction

DNA extraction is completed using Qiagen DNeasy™ Tissue Kits following the procedure outlined in the Qiagen DNeasy™ Tissue Kit Handbook for Cultured Animal Cells. However, omit the first step in the procedure. Samples from suspect tissue cell culture are filter with a 45 µm syringe filter. Add 500 µL of filtered sample to a microcentrifuge tube.

Template DNA should be quantified by a spectrophotometer and 150-300 ng is suggested per reaction (see Appendix A - Analysis of Extracted DNA using an UV Spectrophotometer).

3. PCR Master Mix

A. Materials

10X PCR Buffer
dNTPs (nucleotides)
Primers
Taq polymerase
MgCl2
PCR-grade mineral oil
Thermalcycler
0.5-25 µl & 20-200 µl Pipettors
Gloves (latex or nitrile)
UV cabinet

B. Preparation Guidelines

Reagents for PCR master mix must be mixed and aliquotted in an area designated free of any DNA extracts. A UV hood dedicated to PCR is the best because the low wavelength, high energy UV light will denature any DNA extracts.

a) Prepare bench space using aseptic techniques, always place fresh bench paper over work area. Change gloves after each major step to prevent contamination.
b) Decontaminate work surfaces with 10% chlorine or DNA A*way*™. Tube racks should be soaked in 10% chlorine for 30 minutes.
c) Determine the amount of PCR cocktail needed by summing the total number of samples, and negative and positive controls to be run. Then add enough reagents for 1 more sample to allow for reagent loss during pipetting.
d) Both initial and nested cocktails can be made at the same time, except for adding the primers and Taq. Taq should be added last. Vortex gently by hand.
e) The UV lamp should be left on in the PCR preparation hood for 20-30 minutes prior to and after use.
f) Always use aerosol resistant pipette tips when you are working with PCR reagents and amplified DNA samples

C. Master Mix Formula - Reagent amounts for 50 µl reactions are as follows:

PCR Reagents	**Stock Conc.**	**Final Conc.**	**Volume per reaction tube**	**Example: Volume of 10 rxn**
DNase free sterile water			15.5 µl	155 µl
PCR Buffer (No $MgCl_2$)	10X	1X	5 µl	50 µl
DNTPs	10 mM	0.2 mM	4 µl	40 µl
$MgCl_2$	25 mM	1.5 mM	3 µl	30 µl
TMAC	100 µM	40 µM	20 µl	200 µl
Forward Primer	100 µM	1 µM	0.5 µl	5.0 µl
Reverse Primer	100 µM	1 µM	0.5 µl	5.0 µl
Taq	5 units/µl	2.5 units/rxn	0.5 µl	5.0 µl
DNA sample			1 µl	10-1µl
Total volume			50 µl	500 µl

Note: Aliquot the water, buffer, $MgCl_2$, nucleotides, first for both rounds. The primers and Taq must be added right before amplification, it is not to be frozen when mixed in the master mix.

D. Primers

Largemouth Bass Virus	DNA Sequence 5' to 3'
Forward	GAC TTG GCC ACT TAT GAC
Reverse	GTC TCT GGA GAA GAA GAA

4. Amplification

a.) Wear clean gloves to prevent contamination.
b.) Use same sterile techniques as outlined under General Laboratory Procedures.
c.) Add pre-determined amount of template DNA** to the reaction tube.
d.) Dispense the DNA samples into the reaction tubes in a laminar flow hood to avoid contaminating the lab (the blower need not be running).
e.) The positive control should be dispensed last to minimize handling.
f.) Always add the DNA sample to the master mix.
g.) Add 40 µl of mineral oil to each tube and place in thermalcycler. Run the designated program. See below for program details.
h.) After the 1st and 2nd rounds of amplification have been completed, the PCR product can be run by gel electrophoresis.

Largemouth Bass Virus amplification program - 30 cycles:

Pre-dwell at 94°C	5: 00 minutes
Denature at 94°C	1:00 minute
Anneal at 45°C	1:00 minute
Elongate at 60°C	2:00 minutes
Post-dwell 72°C	2:00 minutes
Hold chilled 4°C	

5. Gel electrophoresis

A. Materials and Reagents

1X TBE Buffer (found in cabinet behind thermalcycler in the Virology laboratory)
1.5% Agarose gel (found in cabinet behind thermalcycler in the Virology laboratory)
6X loading dye (found in -20°C freezer in the Virology laboratory)
DNA ladder standard (found in -20°C freezer the Virology laboratory)
Ethidium Bromide (1 $^{\mu l}/_{ml}$) (found in hood in the Histology laboratory)
UV lamp (found on right counter in the Histology laboratory)

B. Procedure

a.) Agarose gel* can be pre-made in larger volumes and stored in a container that is microwavable. The agarose can be melted by microwaving as needed.
b.) Pour the proper amount of agarose into the gel box and allow at least 30 minutes to cool.
c.) When pouring the agarose the comb should be set up so the DNA will run to the positively charged electrodes ("run to red").
d.) Add appropriate volume of 1X TBE buffer** to unit chamber. Buffer should cover the gel with ~1-2 mm of buffer over the surface of the gel.
e.) Aliquot out 2 µl of loading dye per sample on a clean section of parafilm.

f.) The Gibco 100 bp DNA ladder is used to judge the size of the positives. The ladder has bands at 100-1500 bp, with a brighter band at 600 bp. **The Ladder already contains loading dye.**
g.) Using a pipet aspirate at least 8 µl of sample, mix with the loading dye and carefully place into the gel well.
h.) Run the gel at approximately 75 volts for 1.5 hours.
i.) Place gels in a staining dish (we use a covered "tupperware-like" storage container) and cover with ethidium bromide stain***.
j.) Stain the gels for 20 minutes, return the ethidium bromide to the container, and rinse gels with cold water.
k.) Read gel on UV transilluminator and photograph.
l.) Gel is to be disposed of in tub under flow hood so it can dry out.

* Agarose gel is made up in a 1.5% solution. To make 500 ml of agarose, add:
a.) 500 ml 1X TBE (100 ml 5X TBE (stock) and 400 ml deionized water)
b.) 7.5 grams of DNA grade agarose (high melting)
c.) Heat solution in microwave oven until the agarose goes into solution.
5X TBE and DNA grade agarose can both be found in the PCR equipment cabinet in the Bacteriology laboratory.

**1X TBE buffer:
a.) Just 100 ml of 5X TBE (stock) and 400 ml distilled water.

*** Ethidium bromide is a mutagenic agent that is possibly carcinogenic, great care must be taken when using it. Never touch gels, staining trays, or spatula without gloves and always remove gloves when finished.

C. Dilution Protocol for Gibco DNA Ladder

Mix prior to use:

450 µl of TE buffer (10 mM Tris (HCl), 1mM EDTA, 20 mM NaCl)
50 µl of DNA ladder from manufacturer
100 µl of 6X loading dye
600 µl of DNA standard

D. Results

Largemouth Bass virus:
Final product band = 495 bp

VII. References

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Appendix A - Analysis of Extracted DNA using an UV Spectrophotometer

A. Turn on Gene Quant spectrophotometer before using (no need to let it warm up).

B. Dilute Qiagen DNA isolation 1:100 in order to make a 1:100 dilution (add 5µl of isolate to 495µl of sterile water).

C. Take Reference Measurement first*.

1.) Add 100µl of sterile water to cuvette. Check that there are no bubbles or meniscus visible in the optical path window. Wipe sides of cuvette with lens paper.
2.) Hit [Set Ref] button. Screen should say " Please wait ".
3.) Wait for tone. Tone sounds, screen will display " Insert reference ". Quickly insert cuvette into slot.
4.) Wait for the second tone and remove cuvette when it sounds and screen displays "Remove reference".
5.) Once removed, display should show: "Absorbance": 260nm 0.000AU
6.) Empty cuvette onto paper towel by gently knocking it upside down. Dry cuvette with canned air (keep air can level to prevent spraying liquid into cuvette).

*Note: Once reference is set, there is no need to set it again (i.e. between samples).

D. Sample Measurement

1.) Add 100 µl of dilution sample to cuvette; check that there are no bubbles or meniscus in optical path, wipe sides with Kim-wipe.
2.) Press [Sample] button. Screen will display: " Please wait."
3.) When tone sounds, insert sample. Screen will display: "Insert sample."
4.) When second tone sounds, remove cuvette. Screen will read: "Remove sample."
5.) Screen will automatically show Absorbance at 260nm.
6.) Press the [RNA / DNA] button. Screen will now show: "dsDNA conc 1"
X is the sample conc. in µg/ml ***X*** µg/ml
RECORD THIS NUMBER ON DATA SHEET!
7.) Press the [Select] button. Screen should now read:
"dsDNA conc 2"
X µg/µl ***X*** is the conc. in µg/µl.
RECORD THIS NUMBER ON DATA SHEET!
8.) If reading multiple samples, rinse cuvette with sterile water at least 3 times, then dry with canned air.
9.) For next sample repeat from step one of sample measurement.

E. Calculate the amount of Qiagen isolate sample to add per PCR reaction tube.

*Note: Target amount of DNA per rxn tube is ~ 300ng.

FIRST LOOK AT THE TWO NUMBERS FOR CONCENTRATIONS

The range for dsDNA conc. 1 is: 1-4,000 µg/ml
The range for dsDNA conc. 2 is: 0.001-0.2 µg/µl

Decide which of your two readings best fits within its range, use that conc. number.

1.) Take your concentration "***X***" number and multiply it by the dilution factor used.

(***X***) x dil. factor = actual conc. in tube.

*** Note**: **If DNA sample is too dilute** to measure at 1:200, **make a less dilute solution** for spec analysis. For example - make a 1:50. Record this dilution factor on worksheet.

2.) **Use this formula for dsDNA conc. #2**:

$$\frac{300\text{ng} \;|\; 1\mu\text{l} \;|\; 1\mu\text{g}}{|\ (\text{dil. factor}) \times \boldsymbol{X} \;|\; 1000\text{ng}} = \#\text{ of } \mu\text{l of DNA isolate to add per rxn tube.}$$

3.) **Use this formula for dsDNA conc. #1**:

$$\frac{300\text{ng} \;|\; 1\text{ ml} \;|\; 1\mu\text{g} \;|\; 1000\mu\text{l}}{|\ (\text{dil. factor}) \times \boldsymbol{X} \;|\; 1000\text{ng} \;|\; 1\text{ml}} = \#\text{ of } \mu\text{l of DNA isolate to add per rxn tube.}$$

4.) **Remember if the sample is at a 1:200 dilution then the dilution factor is 200.**

RECORD THE AMOUNT TO ADD PER RXN TUBE ON DATA SHEET!

***** In case this amount to add is less than 0.5µl you can make small dilutions of the sample DNA with sterile water to get a number between 1µl - 10µl.

F.) Shutdown

1.) Clean cuvette with sterile water and dry it with canned air, place cuvette in storage case.
2.) Shut machine off.

*Note: It is safe to keep machine on all day. The deuterium lamp goes into standby mode which, does **not** shorten its life. Reference taken earlier in the day is still good after it is left in standby mode.